

The invention further encompasses modifications which may be made in or immediately adjacent to the loop region between α helices 5 and 6 of a Cry1C protein. This loop region extends from about amino acid 177 to about amino acid 184, with the adjacent amino acids extending from about amino acid 174 to about amino acid 176, and from about amino acid 185 to about amino acid 187.

Another aspect of the invention encompasses modifications in the amino acid sequence which may be made in or immediately adjacent to the loop region between α helices 6 and 7 of a Cry1C protein. This loop region extends from about amino acid 218 to about amino acid 221, with the adjacent amino acids extending from about amino acid 215 to about amino acid 217, and from about amino acid 222 to about amino acid 224.

In a similar fashion, the invention also encompasses modifications in the amino acid sequence which may be made in or immediately adjacent to the loop region between α helix 7 of domain 1 and β strand 1 of domain 2 of a Cry1C protein. This loop region extends from about amino acid 250 to about amino acid 259, with the adjacent amino acids extending from about amino acid 247 to about amino acid 249, and from about amino acid 260 to about amino acid 262.

In addition to modifications of Cry1C peptides, those having benefit of the present teaching are now also able to make mutations in the loop regions of proteins which are related to Cry1C structurally. In fact, the inventors contemplate that any crystal protein or peptide having helices which are linked together by loop regions may be altered using the methods disclosed herein to produce crystal proteins having altered loop regions. For example, the inventors contemplate that the particular Cry1 crystal proteins in which such modifications may be made include the Cry1A, Cry1B, Cry1C, Cry1D, Cry1E, Cry1F, Cry1G, Cry1H, Cry1I, Cry1J, and Cry1K crystal proteins which are known in the art, as well as other crystal proteins not yet described or characterized which may be classified as a Cry1 crystal protein based upon amino acid similarity to the known Cry1 proteins. Preferred Cry1 proteins presently described which are contemplated by the inventors to be modified by the methods disclosed herein for the purpose of producing crystal proteins with altered activity or specificity include, but are not limited to Cry1Aa, Cry1Ab, Cry1Ac, Cry1Ad, Cry1Ae, Cry1Ba, Cry1Bb, Cry1Bc, Cry1Ca, Cry1Cb, Cry1Da, Cry1Db, Cry1Ea,

Cry1Eb, Cry1Fa, Cry1Fb, Cry1Hb, Cry1Ia, Cry1Ib, Cry1Ja, and Cry1Jb crystal proteins, with Cry1Ca crystal proteins being particularly preferred.

Modifications which may be made to these loop regions which are contemplated by the inventors to be most preferred in producing crystal proteins with improved insecticidal activity include, but are not limited to, substitution of one or more amino acids by one or more amino acids not normally found at the particular site of substitution in the wild-type protein. In particular, substitutions of one or more arginine residues by an alanine, leucine, methionine, glycine, or aspartic acid residues have been shown to be particularly useful in the production of such enhanced proteins. Likewise, the inventors have demonstrated that substitutions of one or more lysine residues contained within or immediately adjacent to the loop regions with an alanine residue produce mutant proteins which have desirable insecticidal properties not found in the parent, or wild-type protein. Particularly preferred arginine residues in the Cry1C protein include Arg86, Arg148, Arg180, Arg252, and Arg253, while a particularly preferred lysine residue in Cry1C is Lys219.

Mutant proteins which have been developed by the inventors demonstrating the efficiency and efficacy of this mutagenesis strategy include the Cry1C-R148L, Cry1C-R148M, Cry1C-R148D, Cry1C-R148A, Cry1C-R148G, and Cry1C-R180A strains described in detail herein.

Disclosed and claimed herein is a method for preparing a modified crystal protein which generally involves the steps of identifying a crystal protein having one or more loop regions between adjacent α -helices, introducing one or more mutations into at least one of those loop regions, or alternatively, into the amino acid residues immediately flanking the loop regions, and then obtaining the modified crystal protein so produced. The modified crystal proteins obtained by such a method are also important aspects of this invention.

According to the invention, base substitutions may be made in the *cry1C* nucleotide sequence in order to change particular amino acids within or near the predicted loop regions of Cry1C between the α -helices of domain 1. The resulting Cry1C* proteins may then be assayed for bioinsecticide activity using the techniques disclosed herein to identifying proteins having improved toxin activity.

As an illustrative embodiment, changes in three such amino acids within the loop region between α -helices 3 and 4 of domain 1 produced modified crystal proteins with enhanced insecticidal activity (Cry1C.499, Cry1C.563, Cry1C.579).

As a second illustrative embodiment, an alanine substitution for an arginine residue within or adjacent to the loop region between α -helices 4 and 5 produced a modified crystal protein with enhanced insecticidal activity (Cry1C-R148A). Although this substitution removes a potential trypsin-cleavage site within domain 1, trypsin digestion of this modified crystal protein revealed no difference in proteolytic stability from the native Cry1C protein.

As a third illustrative embodiment, an alanine substitution for an arginine residue within or adjacent to the loop region between α -helices 5 and 6, the R180A substitution in Cry1C (Cry1C-R180A) also removes a potential trypsin cleavage site in domain 1, yet this substitution has no effect on insecticidal activity. Thus, the steps in the Cry1C protein mode-of-action impacted by these amino acid substitutions have not been determined nor is it obvious what substitutions need to be made to improve insecticidal activity.

Because the structures for Cry3A and Cry1Aa show a remarkable conservation of protein tertiary structure (Grochulski *et al.*, 1995), and because many crystal proteins show significant amino acid sequence identity to the Cry1C amino acid sequence within domain 1, including proteins of the Cry1, Cry2, Cry3, Cry4, Cry5, Cry7, Cry8, Cry9, Cry10, Cry11, Cry12, Cry13, Cry14, and Cry16 classes (Table 1), now in light of the inventors' surprising discovery, for the first time, those of skill in the art having benefit of the teachings disclosed herein will be able to broadly apply the methods of the invention to modifying a host of crystal proteins with improved activity or altered specificity. Such methods will not only be limited to the crystal proteins disclosed in Table 1, but may also be applied to any other related crystal protein, including those yet to be identified, which comprise one or more loop regions between one or more pairs of adjacent α -helices.

In particular, such methods may be now applied to preparation of modified crystal proteins having one or more alterations in the loop regions of domain 1. The inventors